

Use of ^{51}Cr -Labeled Mononuclear Cells for Measuring the Cellular Immune Response in Mouse Lungs†

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Spleen cells labeled with ^{51}Cr were used in sensitized syngeneic mice to measure the degree of mononuclear cell infiltration into antigen-challenged tissues. With this method, increased cellular infiltration was found after footpad challenge of mice sensitized with sheep erythrocyte, *Escherichia coli*, and BCG antigens. Cellular response also was determined by using this technique in the lungs of mice sensitized with sheep erythrocytes and BCG. This procedure offers the opportunity to measure cellular infiltration, whether due to cellular or humoral influences, in tissues not easily accessible to conventional immunological manipulation.

The lungs, by virtue of their constant exposure to the environment and their large surface area, are especially vulnerable to substances in inhaled air. Exposure to some of these substances can lead to infections, hypersensitivity reactions, and neoplasms (1, 8). The susceptibility of lungs to injuries of these types is dependent, to a large degree, on the responsiveness of the immunological system. This responsiveness can be compromised by a variety of toxic compounds, often the products of agricultural or industrial activity. Thus, to understand pulmonary defenses against infections, neoplasms, tissue-destructive hypersensitivities, and the degree these defenses can be compromised by toxic substances, it is important to increase knowledge of lung-associated immune responses.

Although considerable interest has been evident in immune responses in the respiratory tract and the lungs (1-3, 7, 11, 14, 15), this information has been difficult to obtain due to the anatomical complexity of the lungs and their relative inaccessibility to the available immunological manipulations (8). Strides made in the understanding of lung-associated immune mechanisms have been limited, to a great degree, to the humoral immune part of the response (8). Cellular immune responses, although of great importance, have been difficult to study in the lungs due to the inaccessibility of the tissues for direct measurements of hypersensitive responses and difficulty in obtaining pulmonary lymphoid cells in adequate numbers for studies of cellular immune responses, especially in mice. In an attempt to obtain quantitative information on cellular immune responses in mouse lungs, we

adapted a technique utilizing ^{51}Cr -labeled lymphocytes previously used to measure delayed-type hypersensitivity (DTH) in footpads (12).

MATERIALS AND METHODS

Animals. Female BALB/c mice (15 to 26 weeks old and weighing 20 to 25 g) were obtained from Flow Laboratories, Dublin, Va., and were matched by age for each experiment. They received pelleted mouse food and water ad libitum.

Antigens. Sheep erythrocytes (SRBC) were obtained from sheep maintained in the Department of Veterinary Science. *Escherichia coli* strain 055:K59:NM (055:B5) was grown, and a suspension of heat-killed bacteria was prepared as described previously (16). *Bacillus Calmette-Guérin* (BCG), strain 1011 Pasteur, was obtained from The Trudeau Institute, Saranac Lake, N.Y., and was grown on IUTM medium (Difco Laboratories, Detroit, Mich.). Tubersol 250, a purified protein derivative of tuberculin (PPD) preparation (100 U of PPD per μg), was obtained from Connaught Laboratories Ltd., Willowdale, Ontario, Canada.

Sensitization. Various immunization regimens were used to produce sensitization, depending on the antigens being used. The same methods were used however, irrespective of whether a footpad or pulmonary challenge was done later. Sensitization of mice with SRBC was done by the method of Schwartz et al. (12). Briefly, 0.2 ml of a 0.01% freshly washed suspension of SRBC was given intravenously 4 days before challenge. The same regimen was also used for sensitization with dried whole *E. coli* antigen, using a dose of 1 μg in 0.2 ml of saline. Sensitization with BCG was accomplished by the method of Crowle (4). BCG harvested from 3- to 5-week-old broth cultures was washed in saline and suspended at 10 mg (wet weight) per ml in the aqueous phase of an emulsion made with incomplete Freund adjuvant (Difco). Sensitization was done by injecting 0.1 ml of the emulsion subcutaneously in the inner thigh twice at a 1-week interval (5). The BCG-sensitized mice were challenged 21 days after the second injection.

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Determination of cellular immunity by ^{51}Cr incorporation. Unsensitized syngeneic mice were used to provide lymphoid cells for ^{51}Cr labeling. Spleens from several mice were aseptically removed and pooled, and a single cell suspension was made by mincing the tissues with scissors in cold RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.), followed by repeated aspiration through an 18-gauge needle. Clumps of cells were removed by a single passage through a 21-gauge needle. The cell suspension was depleted of erythrocytes by treatment with 0.84% tris(hydroxymethyl)aminomethane-buffered ammonium chloride for 10 min at room temperature, followed by three washes with cold RPMI 1640. The cell suspension was adjusted to 10^8 cells per ml, and 40 μCi of ^{51}Cr (sodium chromate, 6.7 Ci/mmol, New England Nuclear Corp., Boston, Mass.) was added per ml of suspension. Incubation for 45 min at 37°C was followed by three washes with cold RPMI 1640 to remove unincorporated label. The suspensions were adjusted to a concentration of 5×10^7 cells per ml of RPMI 1640, and 0.2 ml was injected intravenously 3 h before antigenic challenge of the sensitized mice.

Antigenic challenge. Footpad responses were determined by intradermal injections of 0.02-ml volumes of antigens into the plantar area. The concentrations of antigens were as follows: SRBC, 20% suspension; *E. coli*, 100 $\mu\text{g}/\text{ml}$; BCG, 1.5 mg/ml; PPD, 100 U/ml.

For intratracheal injections, the mice were anesthetized with pentobarbital sodium, and an incision was made just posterior to the intermandibular space extending 1 cm caudal to the sternum. The trachea was exposed, and 0.05 ml of antigen suspension was introduced into the trachea with a 27-gauge needle. Control animals were injected in the identical manner with sterile saline only.

Determination of response. At 48 h after challenge injections (9) the lungs and feet were removed and washed of excess external blood by dipping in saline, and the radioactivity was determined with a gamma scintillation counter. The radioactivity present in each tissue was expressed as the percent incorporation of the total radioactivity injected. Comparison of groups was done by a one-way analysis of variance and the Newman-Keuls sequential range test (13).

Histology. Lungs from control and test mice were removed in toto and perfused through the trachea with phosphate-buffered Formalin (pH 7.2). Paraffin-embedded sections (6 μm) were prepared and stained with hematoxylin and eosin.

RESULTS

The degree of sensitization produced in mice after intravenous immunization with SRBC was tested by challenge with SRBC in the footpads. A significant increase in ^{51}Cr -labeled cells was obtained in the footpads, a finding similar to those reported by Schwartz et al. (12) (Fig. 1).

Since many of our studies include humoral responses to *E. coli* antigens, mice were injected intravenously with 1.0 μg of *E. coli* cells. Subsequent challenge in the footpads of the sensitized mice resulted in an increased ^{51}Cr presence in the injected tissues. However, a smaller in-

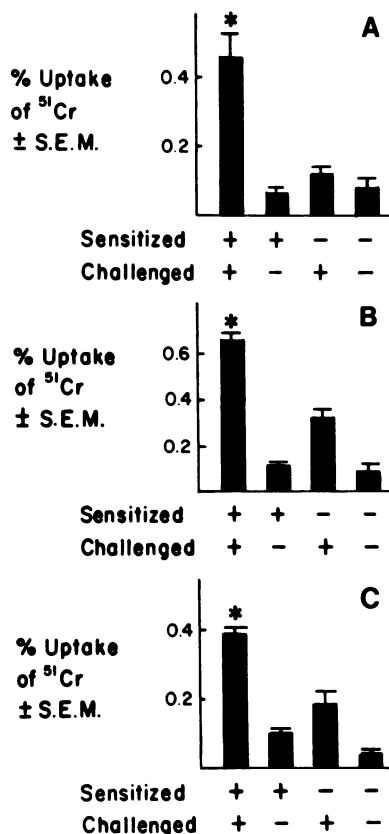


FIG. 1. Uptake of ^{51}Cr -labeled cells in the feet of sensitized mice. Groups of four mice were immunized with SRBC (A), *E. coli* (B), or BCG (C) or were given saline injections. At 4 or 21 days (BCG) after injection, mice were injected intravenously with ^{51}Cr -labeled syngeneic spleen cells and given footpad challenge injections. Feet were removed 48 h later, and radioactive label present was measured. S.E.M., Standard error of the mean; *, significant as $P < 0.01$ compared with other experimental groups.

crease of ^{51}Cr -labeled cells was also found in the footpads of challenged, unsensitized mice, indicating a possibility that a nonspecific inflammatory mechanism contributes to the accumulation of ^{51}Cr -labeled cells in response to this antigen. Similar results were obtained with BCG (Fig. 1).

Other antigen and injection combinations were used to produce cellular immune responses in the lungs. The antigens used for sensitization in these tests were SRBC and BCG. *E. coli* antigen was tried but discarded since preliminary tests in the lungs indicated a nonspecific inflammatory cell accumulation similar to that found in the footpads. Intratracheal challenge with SRBC was done on SRBC-sensitized animals, and that with BCG and PPD was done on BCG-sensitized mice.

In these series of experiments, all procedures produced responses, but the combination of subcutaneous injection with BCG followed by intratracheal challenge with PPD produced the best responses. To achieve this pulmonary sensitivity, mice were injected subcutaneously with 1 mg of BCG twice at a 7-day interval. Three weeks after the second injection, the sensitized mice and appropriate controls were injected first with ^{51}Cr -labeled spleen cells, followed by intratracheal injections of PPD. For SRBC sensitization, the mice were injected intravenously 4 days before challenge. Table 1 summarizes one series of studies where responses in both lungs and footpads were determined. Figure 2 summarizes the results of a second set of experiments, using only intratracheal challenges with PPD and SRBC. All challenges except PPD injection in the footpads resulted in a significantly higher accumulation of ^{51}Cr label in tissues of sensitized animals.

Hematoxylin- and eosin-stained lung tissue from control and sensitized, challenged animals was examined histologically. In contrast to the lungs of the control mice, which were histologically normal, the lungs of the sensitized mice had significant histological changes. These changes primarily consisted of perivascular edema and accumulations of leukocytes which were predominantly lymphocytes and monocytes. The predominant perivascular change was associated with pulmonary venules and veins. A

TABLE 1. Uptake of ^{51}Cr -labeled spleen cells in the lungs and feet of sensitized mice

Sensitization ^a	Challenge ^b	% Uptake of ^{51}Cr	
		Lungs	Feet
SRBC	SRBC	1.57 ± 0.12^d	0.37 ± 0.08^e
	Saline	1.13 ± 0.14	0.17 ± 0.02
BCG	BCG	2.45 ± 0.14^e	0.26 ± 0.03^e
	Saline	1.82 ± 0.05	0.19 ± 0.04
BCG	PPD	2.87 ± 0.16^e	0.07 ± 0.01
	Saline	1.62 ± 0.10	0.07 ± 0.01

^a Sensitized with intravenous SRBC or subcutaneous BCG in incomplete Freund adjuvant.

^b Amounts were as follows: SRBC, 0.05 ml of a 50% suspension in the lungs or 0.02 ml of a 20% suspension in the footpad 4 days after sensitization; BCG, 1.0 mg in the lungs or 0.03 mg in the footpad 21 days after sensitization; PPD, 50 U in the lungs or 2 U in the footpad 21 days after sensitization.

^c Average of four to six mice per group \pm the standard error.

^d Significant at $P < 0.05$ when compared with the control group.

^e Significant at $P < 0.01$ when compared with the control group.

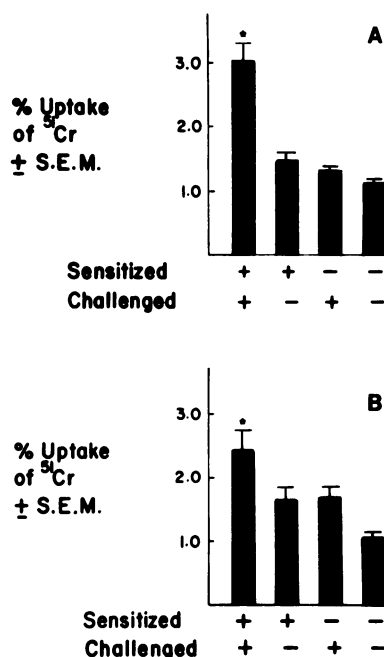


FIG. 2. Uptake of ^{51}Cr -labeled spleen cells in lungs of sensitized mice. Groups of four or five mice were immunized with BCG (A) or SRBC (B) or were given saline injections. (A) At 21 days after immunization with BCG, mice and controls were injected intravenously with ^{51}Cr -labeled syngeneic spleen cells and challenged intratracheally with 50 U of PPD. (B) At 4 days after immunization with SRBC, mice and controls were injected intravenously with ^{51}Cr -labeled syngeneic spleen cells and challenged intratracheally with 0.05 ml of a 50% SRBC suspension. At 48 h after challenge, lungs were removed, and radioactive label present was measured. S.E.M., Standard error of the mean; *, significant at $P < 0.05$ compared with other experimental groups.

few granulocytes were interspersed in the lymphomononuclear accumulations, and occasionally these were concentrated in distinct form.

DISCUSSION

Cell-mediated immune responses are responsible for resistance to infections by intracellular microorganisms (bacteria and viruses), rejection of tissue cells (allografts and neoplasms), graft versus host reactions, and DTH. Thus, cell-mediated immune responses may contribute greatly to the lung's ability to resist infection and neoplastic growth and may determine the intensity of hypersensitive reactions in these tissues.

Cellular immunity, including DTH, has been studied in the lungs of various animal species. Local cellular and humoral immunity to influenza was studied in guinea pigs (15), whereas cell-mediated resistance to aerogenic infection

with *Listeria monocytogenes* was determined in mice (14). Macrophage migration inhibition in the presence of antigen was examined in lung lymphocytes by Henney and Waldman (7). DTH in the lungs has been the focus of several other studies (3, 4, 6, 8, 10, 11). A variety of methods were used to assess both the development of DTH and the consequence of this sensitivity both in resistance to infections and lesion development. In most of these studies, the methods that were used lacked the precision usually available when a specific cell population and its function can be determined quantitatively, as is the case in studies of humoral immunity. The method for the quantification of the immune response in the lung presented in this paper is probably more sensitive than other methods currently available. A further advantage of this procedure is that it measures the accumulation of an indicator population of mononuclear cells at the site of the challenge. The accumulation of mononuclear cells is part of the mechanism of DTH. The sensitivity of the assay will probably be improved as the optimum conditions for sensitization and challenge and the optimum number of indicator cells required are determined.

Two antigens were used successfully for the induction of pulmonary sensitivity. BCG induced a degree of sensitization which could be measured readily after challenge with either BCG or PPD. There was evidence, however, that the inflammatory properties of BCG in lungs also caused nonspecific cell accumulation; therefore, PPD was judged to be a better agent for challenge.

The method presented in this paper is quantitative and sensitive. With appropriate controls, it should differentiate between specific hypersensitive reaction and inflammatory cell accumulation in response to irritant properties of the antigen. It is possible that the cell accumulation in response to challenge may not be strictly a DTH response, but may include antibody-dependent reactions much the same as the Arthus phenomenon. The likelihood that immune complex reaction is important was diminished by sensitization techniques which induce primarily

DTH and by the measurement of mononuclear rather than neutrophil infiltration. Though syngeneic cells were used in this study, in larger animal species the use of autochthonous cells is a possibility. Such modification would have the advantage of allowing this method to be used in species where syngeneic donor cell sources are not available.

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